

## REMARKS

### The Office Action

Claims 1-25 are pending in this case. Claims 9, 10, and 25 stand rejected, under 35 U.S.C. § 112, second paragraph, and claims 1, 2-6, 9, 10, and 21-24 stand rejected, under 35 U.S.C. § 103. These rejections are addressed below.

### Claim Amendments

Claims 9, 10, and 25 have been amended to incorporate the limitations of claim 1. These amendments find support in claim 1 and in the specification, for example, at page 15, lines 13-15. In addition, claims 5 and 22 have been amended for the purpose of clarification. No new matter is added by these amendments.

### Rejections under 35 U.S.C. § 112

Claims 9, 10, and 25 stand rejected under 35 U.S.C. § 112, second paragraph. This rejection may be withdrawn. As requested by the Office, Applicants have added to these claims the step of introducing an adenoviral vector to a pigment epithelial cell and have incorporated the language of product claim 1.

### Rejections under 35 U.S.C. § 103(a)

Claims 1, 2, 4-6, 9, and 21-24 stand rejected, under 35 U.S.C. § 103(a), as being unpatentable over Reichel et al. Claims 1 and 3 stand rejected, under 35 U.S.C. § 103(a), as being unpatentable over Reichel et al. in combination with Kovesdi. Claims 1 and 10 stand rejected, under 35 U.S.C. § 103(a), as being unpatentable over Reichel et al. in combination with Tezel. And claims 1 and 24 stand rejected, under 35 U.S.C. § 103(a), as being unpatentable over Reichel et al. in combination with Funk, Williams, and Tezel.

These rejections are respectfully traversed because the primary reference, Reichel,

rather than supporting a case of *prima facie* obviousness teaches away from the claimed invention. Each of Applicants' claims requires a pigment epithelial cell of the eye that comprises vector DNA of an adenoviral vector expressing a nucleic acid and having no adenoviral coding DNA sequence. This type of vector, as stated in the present specification at page 6, lines 1-4 and as demonstrated at pages 33-35 and 40, allows for long-term expression in the pigment epithelium of the eye. Reichel does not disclose such a vector and if anything indicates that high capacity adenoviral vectors are incapable of supporting long-term gene expression in this tissue.

As indicated previously in this case, Reichel is a review article summarizing the state of the art at the time of its publication. It describes a number of gene transfer systems utilized for ophthalmologic applications, some of which involve adenoviral vectors. In particular, at page 7, a *non high capacity* adenoviral vector is described for gene transfer into the eye. This vector fails to suggest Applicants' claimed invention. First, the vector includes viral sequence and so falls outside of Applicants' claims. Moreover, after transfer into the retinal pigment epithelium there was only short-term expression, "limited to about 3 weeks." This also differs from Applicants' claimed vectors, which allow for long-term gene expression in this tissue.

On page 8, another system is described in which EAMs, vectors that resemble high capacity adenoviral vectors are utilized. The publication being reviewed, Kumar-Singh et al., *Hum. Mol. Genet.* 7:1893 (1998), is attached. As indicated in Kumar-Singh and Applicants' specification at page 4, line 11- page 5, line 2, following subretinal injection of the EAM, gene expression was detected exclusively in the neuronal part of the retina, and not in the retinal pigment epithelium. For this reason alone, Reichel cannot suggest the presently claimed invention.

Moreover, although neuronal cells are post-mitotic and can no longer divide, expression of the PDE gene carried by the Kumar-Singh EAM vector was only transient. As demonstrated in Kumar-Singh, by several methods (RT-PCR, Western Blot analysis,

and determination of PDE activity), it was shown that, while there existed some minor detectable transcript at 18 weeks post-injection , PDE protein was no longer detectable after 90 days and PDE activity had begun to decrease by 28 days. This reference therefore highlights the nonobviousness of Applicants' claimed invention. Despite the fact that the neuronal retina is a cell layer immediately adjacent to the retinal pigment epithelium and as stated above is composed of post-mitotic cells as is the retinal pigment epithelium, expression in this tissue was only short-term. Thus, Reichel not only fails to suggest that high capacity adenoviral vectors can be used for long-term expression in pigment epithelium of the eye, this reference actually teaches away from the claimed invention by indicating that long-term expression after gene transfer by a high capacity adenoviral vector into a very similar tissue could not be achieved and certainly would not be expected for related vectors. Reichel provides no motivation for the use of an adenoviral vector as presently claimed to produce the pigment epithelium cells of the present invention. Moreover, as none of the secondary references remedies this deficiency in the Reichel reference, the rejections based on Reichel alone as well as Reichel in combination with with Kovesdi, Tezel, Funk or Williams should be withdrawn.

#### Information Disclosure Statement

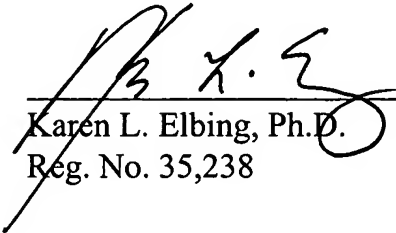
Applicants note that the Form PTO 1449 that was submitted with an Information Disclosure Statement filed on October 16, 2002 has not been initialed and returned, and hereby request that it be initialed and returned with the next Action.

## CONCLUSION

Applicants submit that the claims are now in condition for allowance, and such action is respectfully requested. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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# Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: application to the rescue of photoreceptor degeneration

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First ( $\Delta E1/E3$ ) and second ( $\Delta E1+\Delta E2/E3/E4$ ) generation adenovirus (Ad) vectors have been shown previously to be of limited use in the treatment of human genetic diseases due to the induction of a host cytotoxic T-cell mediated immune response against virally expressed genes. In addition, a limited cloning capacity of ~8 kb does not cater for the incorporation of large upstream sequences essential for regulated tissue-specific expression or inclusion of multiple gene-expression cassettes. In this study we have exploited our recently developed Ad-based vector, the encapsidated adenovirus mini-chromosome (EAM) from which all of the viral genes have been deleted. EAMs contain only the inverted terminal repeats required for replication and five *cis*-acting Ad encapsidation signals necessary for packaging. We have shown previously that EAMs can efficiently transduce a variety of cell types *in vitro*. In this study we demonstrate that EAMs can transduce and rescue cells from the neurosensory retina *in vivo*. EAM-mediated delivery of the  $\beta$  subunit of cyclic GMP phosphodiesterase (PDE) cDNA to mice affected with retinal degeneration (*rd*) allows prolonged transgene expression and rescue of rod photoreceptor cells. RT-PCR analysis from the injected retina indicates that transgene products are present for at least 18 weeks post-injection. Both the  $\alpha$  and  $\beta$  subunits of PDE could be detected up to 90 days postnatal in EAM-injected *rd* retina by western analysis. A maximal PDE activity of 150 nm/min/mg was detected at 33 days postnatal. Examination of outer nuclear thickness showed significant differences up to 12 weeks post-injection. These results demonstrate an improved level of rescue over first-generation adenoviral vectors and suggest

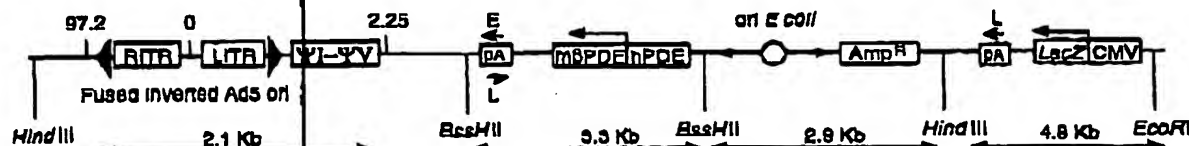
the possibility of successful EAM-mediated treatment of some retinal diseases in humans.

## INTRODUCTION

Adenovirus-mediated gene transfer is a promising technology for the treatment of human genetic diseases. Whereas adenovirus vectors have several advantages over other vector systems, limitations have become apparent with their use *in vivo*. These limitations include short-term expression of the delivered gene and loss of transduced cells. Despite the deletion of genes E1a and E1b in the so-called 'first generation' adenoviral vectors, production of immunogenic viral proteins in transduced cells leads to MHC class I and class II mediated display of viral peptides on the surface of infected cells, targeting these cells for T-cell mediated clearance. Re-administration of adenoviruses to restore transgene expression is inefficient because of the production of neutralizing antiviral antibodies (1,2). In order to overcome some of these problems, 'second generation' viral vectors containing deletions in other regions of the Ad genome (such as E2a and E4) have been developed; however, there is some uncertainty whether these vectors result in improved transgene persistence in animals (3-6). We (7,8) and others (9-13) have recently described a novel class of adenoviral vectors from which almost the entire Ad genome has been deleted [encapsidated adenovirus mini-chromosomes (EAMs)]. Our initial studies indicated that EAMs can be used to transduce cells *in vitro* (7). As our first step towards the use of EAMs *in vivo*, we describe EAM-mediated transduction of cells from the neurosensory retina. In particular, we show that this novel vector system can be used to transduce and rescue degenerating photoreceptor cells in the retinal degeneration (*rd*) mouse, a model for retinitis pigmentosa (RP) in humans.

RP is the name given to an array of disorders characterized by night blindness, progressive constriction of the visual field, an abnormal or non-detectable electroretinogram and photoreceptor degeneration. The disease may be inherited in an autosomal-

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**Figure 1.** Structure of pAd5βPDE. The  $\beta$  subunit of *Mass musculus* cAMP-phosphodiesterase (m $\beta$ PDE) and the *E. coli*  $\beta$ -galactosidase (*LacZ*) cDNAs are regulated by a human  $\beta$ -PDE (hPDE) and cytomegalovirus (CMV) enhancer/promoter, respectively. The  $\beta$ -PDE cDNA and *LacZ* gene are polyadenylated by an SV40 early (E) and late (L) signal, respectively. The bacterial origin of DNA replication (ori *E. coli*) and ampicillin selection (*Amp<sup>R</sup>*) for propagation in bacteria are encoded by pBSBKS (Stratagene). The fused adenovirus origins of replication are represented by a left and right inverted terminal repeat (LITR/RITR). Following replication, encapsidation of DNA into mature capsids is accomplished by the five packaging signals ( $\Psi$ 1- $\Psi$ 5).

dominant, autosomal-recessive, X-linked or digenic manner. Prevalence estimates range from 1 in 3000 to 1 in 6000, making RP one of the most common inherited forms of retinal disorders in humans. Some forms of RP in humans (14,15), mice (16,17) and dogs (18,19) have been associated with mutations in the gene encoding the  $\beta$  subunit of guanosine 3',5'-monophosphate (cyclic GMP) phosphodiesterase (PDE).

A nonsense mutation (17) and/or an *Xmv-28* proviral insertion (20) in the gene encoding  $\beta$ -PDE causes retinal degeneration in the *rd* mouse, resulting in an aberrantly spliced mRNA and/or a predicted truncated protein product (21). Similarly, a nonsense mutation in the  $\beta$ -PDE gene has been found in the Irish Setter dog (19). Homozygous *rd* mice undergo complete degeneration of photoreceptors within the first three weeks postnatal. We have shown previously that expression of  $\beta$ -PDE in transgenic *rd* mice rescues photoreceptor degeneration (22). Moreover, heterozygous *rd* mice enjoy unimpaired vision, indicating that the mutation results in a lack of function, making the *rd* mouse and its equivalent in humans an excellent candidate for somatic gene therapy.

Several groups have demonstrated previously the efficacy of adenovirus as a gene transfer vector for the retina (23-26). In each of these investigations it has been demonstrated that a first-generation adenoviral vector is effective at delivering the *Escherichia coli LacZ* gene regulated by a CMV promoter to the retina. In addition, first generation adenovirus vectors have been used to deliver ciliary neurotrophic factor or a  $\beta$ -PDE cDNA regulated by a CMV promoter to the *rd* retina (27,28). The length of expression and rescue of degenerating *rd* retina using these vectors was transient, lasting up to 6 weeks (28). Nonetheless, these studies demonstrated an important 'proof of principle' for gene therapy of RP. These studies taken together with data from other organ systems suggest that we need to develop and test improved vector systems. In order to further our potential for developing a gene-based therapy for RP, we have constructed an EAM containing the  $\beta$ -PDE cDNA and delivered it to the subretinal space of homozygous *rd* mice. We have determined the expression of the transgene by RT-PCR, western blot analysis and PDE activity assays. Phenotypic rescue of the disease phenotype was determined by histological analyses. Our results indicate that EAMs may provide a significantly improved avenue for treatment of RP when compared with first-generation adenoviral vectors and may eventually contribute towards a gene therapy for RP.

## RESULTS AND DISCUSSION

In contrast to most tissues, Ad-mediated gene therapy for vertebrate photoreceptors is particularly favorable from an immunological perspective. Certain organs such as testis, ovary, pregnant uterus and the eye are immunologically tolerant to foreign antigens, and are considered sites of immune privilege (29). Inflammatory cells entering the anterior chamber of the eye in response to viral infection undergo Fas-ligand induced apoptosis (30). However, a small number of inflammatory cells are associated with virally infected retina or subretinal space, indicating that these particular sites are only immunologically sequestered (30,31). As a consequence, preliminary gene therapy applications using first-generation adenovirus vectors to rescue the *rd* phenotype have resulted in minimal or physiologically insignificant levels of PDE activity. Recently, it has been demonstrated that a first-generation adenoviral vector encoding the  $\beta$  subunit of PDE driven by the CMV promoter can rescue photoreceptors in the *rd* mouse for a period of ~6 weeks (28). We have investigated the use of a vector not able to express any Ad-encoded proteins to determine if the length of transgene expression can be extended with respect to first generation viral vectors. These studies will aid in our efforts to eventually determine an efficacious treatment of retinal diseases.

We have shown previously that a circular molecule containing fused embedded adenovirus inverted terminal repeats (origins of replication) and five packaging elements is replicated and packaged into mature virions in the presence of helper virus. The activity of an embedded origin of replication leads to the generation of linear mini-chromosomes from circular molecules (7). Ad DNA is packaged into virions *in vivo* in a polar, left-to-right fashion and is dependent on *cis*-acting packaging elements. The Ad5 packaging domain extends from nucleotide 194 to 358 and is composed of at least five distinct elements that are functionally redundant (32,33). These mini-chromosomes (34) or EAMs have a theoretical cloning capacity of 36 kb. We have shown previously that EAMs containing up to 22 kb of non-viral exogenous DNA can be used to transfer multiple gene-expression cassettes to cells in culture (7). In this study we have constructed an EAM consisting of a full-length (2.75 kb) murine  $\beta$ -PDE cDNA regulated by a human  $\beta$ -PDE promoter (350 bp), an *E. coli LacZ* gene regulated by a cytomegalovirus promoter and the Ad5 origin of replication together with five *cis*-acting viral packaging signals—pAd5βPDE (Fig. 1).

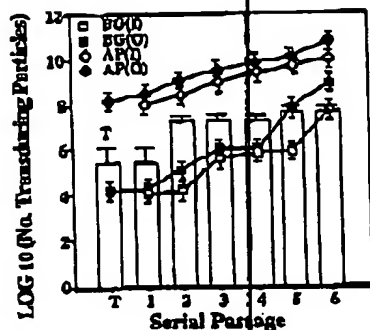


Figure 2. Amplification of Ad5βPDE. Following cotransfection (T) of 293 cells (vertical bars) with 5 µg of pAd5βPDE and 0.5 µg of linear hpAP DNA, six serial passages were performed to amplify both Ad5βPDE and hpAP viruses. Assays detecting β-galactosidase (BG) or alkaline phosphatase (AP) activities were used to determine the total input (I) or output (O) of each type of virus.

Cotransfection of pAd5βPDE with linear adenoviral DNA into human embryonic kidney 293 packaging cells produces EAMs and helper viruses 6–12 days post-transfection, as evidenced by the appearance of a cytopathic effect. The EAMs are propagated and amplified by six serial passages in 293 cells with a *trans*-acting E1/E3-deleted helper virus expressing human placental alkaline phosphatase (hpAP). The choice of hpAP as a helper allows monitoring of the relative populations of helper virus and EAMs at the various stages of serial propagation (Fig. 2). In agreement with previous results, we found that the smaller genome of the EAMs (~13 kb) has a replicative advantage over the helper virus (36 kb) and that over six rounds of replication, there was a net accumulation of EAMs at a rate 100-fold greater than that of the helper virus. Our results indicate that EAMs containing 13 kb of DNA can be propagated to a titer of  $10^{10}$  transducing particles/ml in the presence of hpAP helper virus and that the EAMs can be separated from helper virus on CsCl gradients. Evaluation of virion concentrations in the final EAM preparation before injection indicated a maximal hpAP 'contamination' of 2.8% (data not shown) and a gross EAM structure similar to that of adenovirus (Fig. 3). Southern blot analysis of Ad5βPDE viral DNA reveals the presence of monomers, and dimers in a head-to-head, head-to-tail and tail-to-tail configuration (Fig. 4).

A total of  $3.4 \times 10^7$  purified EAMs (Ad5βPDE) were injected into the subretinal space of homozygous *rd* mice by a *trans*-choroidal approach between 5 and 6 days postnatal, a time-point before the onset of retinal degeneration. The contralateral eye of the same mouse was injected with Ad5βN—an EAM identical in all respects to Ad5βPDE except that it does not contain a β-PDE cDNA.

To determine whether β-PDE was expressed in Ad5βPDE-transduced retina, we examined retinas from injected eyes for production of β-PDE transcripts and for cGMP-PDE activity. Total RNA was extracted from Ad5βPDE and Ad5βN-injected retinas at 2 week intervals until 18 weeks post-injection (the latest time point examined), reverse transcribed and subjected to PCR. RT-PCR amplification resulted in a fragment of 650 bp only in RNA templates prepared from retinas injected with Ad5βPDE. Since the *rd* mouse has an ochre mutation in codon 347, converting TAC to TAA, a new *Dde*I site is created in *rd*



Figure 3. Electron micrograph of negatively stained EAMs exhibiting a gross structure and size identical to that of adenovirus.

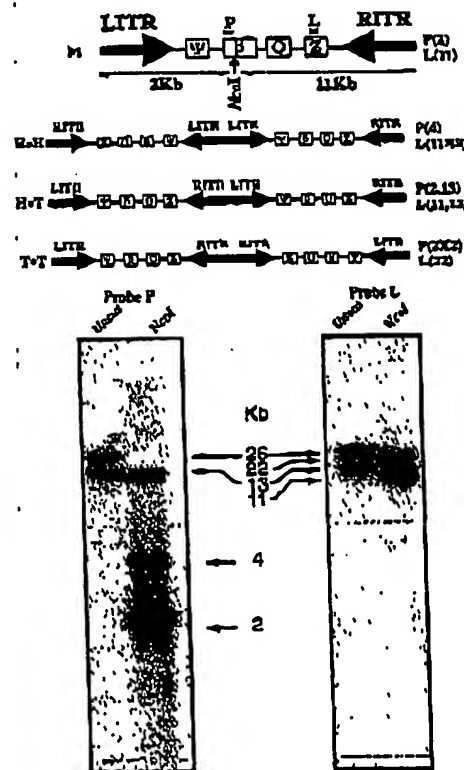
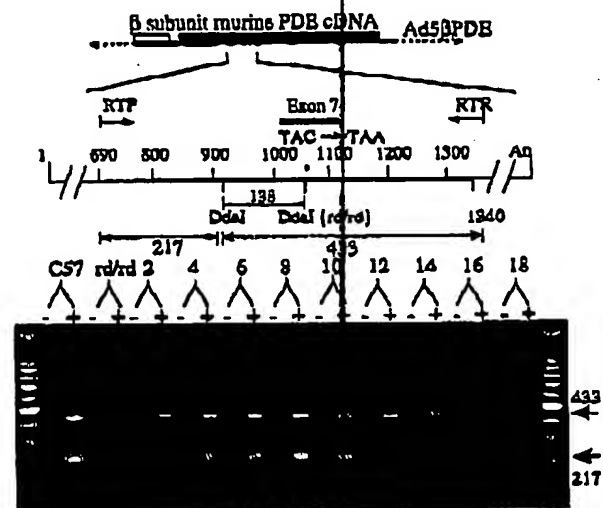


Figure 4. Southern blot analysis of DNA prepared from Ad5βPDE EAMs with probes from the C-terminus of β-PDE (P) or the N-terminus of LacZ (L). Note the presence of monomers (M), head (H)-to-tail (T) dimers, H-to-H dimers and T-to-T dimers. The orientation of the adenoviral packaging signals (Ψ), β-PDE cDNA (β), βSINKS (O) and LacZ gene (wid) respect to the adenovirus left and right inverted terminal repeats (LTR/RTR) in each dimer is shown.

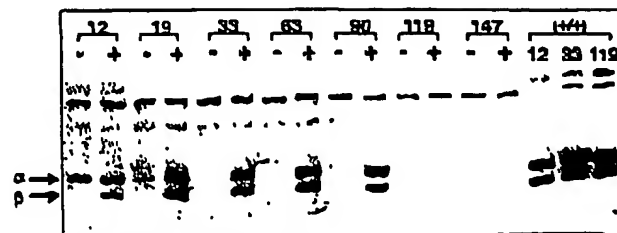


**Figure 5.** RT-PCR amplification of transgene and native  $\beta$ -PDE transcripts from Ad5 $\beta$ PDE transduced *rd* retinas. PCR amplification from cDNA using  $\beta$ -PDE specific primers RTP and RTR results in a 650 bp product encompassing exon 7 of the murine  $\beta$ -PDE gene. Homozygous *rd* mice have been shown previously to contain a *Ddel* restriction site at 1045 in addition to the one at nt 907. RNA templates arising from Ad5 $\beta$ PDE-transduced cells only have one *Ddel* site at position 907. RT-PCR was performed from retinal cDNA prepared from injected mice at various ages post-injection in 2 week intervals up to 18 weeks. RNA was either treated with (+) or without (-) reverse transcriptase prior to PCR.

homozygotes (17). Incubation of the RT-PCR products with *Ddel* followed by fractionation on agarose gels identified digestion products associated only with the transduced gene (Fig. 5). Expression of the  $\beta$ -PDE transcript was still evident at 18 weeks postnatal. To determine whether there was adenovirus-mediated extra-ocular transgene expression in mice receiving subretinal Ad5 $\beta$ PDE injection, we performed RT-PCR in samples of liver, lung, brain and kidney RNA from five randomly selected (injected) mice. No expression of the transgene was detected in any of these samples (data not shown).

We have qualitatively examined expression of the  $\alpha$  and  $\beta$  subunits of PDE by western analysis in Ad5 $\beta$ PDE and Ad5 $\beta$ N-injected *rd* retinas up to 147 days postnatal. The  $\alpha$  subunit of PDE could barely be detected at day 19 postnatal in the Ad5 $\beta$ N-injected *rd* retina. Both the  $\alpha$  and  $\beta$  subunits of PDE could be detected up to 90 days postnatal in Ad5 $\beta$ PDE-injected *rd* retina (Fig. 6) suggesting that some photoreceptor outer segments were still present at this time in the treated animals, since this is the exclusive localization of the rod PDE (35).

We have also measured the activity of cOMP-PDE in Ad5 $\beta$ PDE or Ad5 $\beta$ N-injected *rd* retinas up to 147 days post-injection. Our results indicate that the  $\beta$  subunit of cyclic GMP PDE is processed and forms a functional PDE complex with the  $\alpha$  and  $\gamma$  subunits. A maximal PDE activity of 150 nm/min/mg was detected at 33 days postnatal in Ad5 $\beta$ PDE injected *rd* retina (Fig. 7). This was approximately three times that found in Ad5 $\beta$ N-injected *rd* retina at the same time-point. Although maximal activity levels



**Figure 6.** Western analysis of  $\alpha$ -PDE and  $\beta$ -PDE subunits in Ad5 $\beta$ PDE (+) or Ad5 $\beta$ N-injected (-) *rd/rd* retinas through day 147 postnatal. Untreated age-matched CS7BL (+/+) retinas at days 12, 33 and 119 were used as positive controls. Note that this is an amplified signal and hence not a representation of the stoichiometric ratio of each subunit.

are not equal to those in age-matched CS7BL control mice ( $220 \pm 40$  nm/min/mg), the data are encouraging in that wild-type levels of  $\beta$ -PDE are not required for rescue of the *rd* phenotype. We have observed some variability in PDE activity in age-matched Ad5 $\beta$ PDE-injected *rd* mice. We attribute this to the small size of the eye in 5-day-old *rd* mice and the difficulties in exact reproducibility of the injection between individuals. Nonetheless, the mean PDE activity is clearly greater in Ad5 $\beta$ PDE-injected than in Ad5 $\beta$ N-injected retinas. Furthermore, the PDE activity attained in EAM-injected *rd* retinas is greater than that using first-generation adenoviral vectors (28).

It has been shown previously that the light-activated state of PDE can be mimicked by treating the preformed PDE  $\alpha\beta\gamma$  complex with trypsin, histone, or polycations, such as protamine, which selectively digest or remove the PDE $\gamma$  subunit inhibitory constraint (36). The kinetics of PDE activation *in vitro* correspond to the degradation of PDE $\gamma$  by trypsin (37). Homogenates were prepared from control retinas and from three Ad5 $\beta$ PDE- or Ad5 $\beta$ N-injected *rd/rd* retinas at 12 days postnatal and assayed for PDE activity after treatment with trypsin. Trypsinization markedly increased PDE activity in wild-type mice (22-fold) and Ad5 $\beta$ PDE-injected mice (10-fold) but minimally in Ad5 $\beta$ N-injected or *rd* homozygotes (Fig. 8).

Outer nuclear layer (ONL) thickness is considered a reliable index for comparing photoreceptor cell number between retinas subjected to various traumatic or genetic insults (38). To obtain a quantitative estimate of the possible differences in the number of photoreceptors harbored by our experimental retinas, representative sections taken at different intervals post-injection were scored for ONL thickness. Whereas there are eight to 10 rows of photoreceptor nuclei in mature wild-type mice or heterozygous *rd* mice, there is only a single row of nuclei in homozygous *rd* mice at 17 days postnatal. All photoreceptor nuclei are absent in the *rd* retina by 6 weeks postnatal. Examination of ONL thickness at 2 week intervals in Ad5 $\beta$ PDE or Ad5 $\beta$ N-injected *rd* retinas up to 16 weeks post-injection (the latest time point examined) still showed significant differences up to 12 weeks post-injection (Figs 9 and 10).

Incorporation of the *E. coli* Lac Z gene in our construct has enabled us to determine the total spread of EAMs across the subretinal space. Our data indicate that an area of  $\sim 1$  mm<sup>2</sup> surrounding the injection site is infected by a single EAM injection (Fig. 11) which contains the entire region of resoua. Due



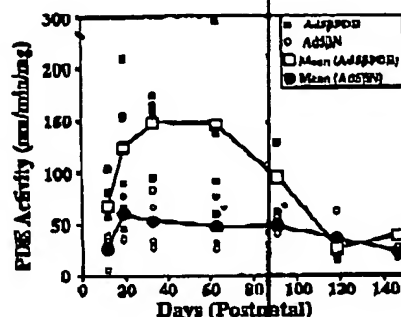


Figure 7. Actual and mean cGMP-PDE activity in *rd/rd* retinas after treatment with Ad5BPDE and Ad5BN. Activities were measured at days 12, 19, 33, 63, 91, 119 and 147 postnatal ( $n = 3-5$  retinas). Data from all retinas are included in the above figure; however, it is unlikely that the two samples indicated by (\*) were injected in the subretinal space (see text).

to the small size of the mouse eye, only single injections were performed in our experimental retinas. In the case of larger eyes such as in humans, multiple injections may be possible.

Finally, although our results are promising, we are attempting to improve the viral system employed in this study further. As there is some contamination of helper virus in our system (albeit with a first generation E1/E3-deleted virus) it is possible that a slow immune response may be generated against virally transduced cells. It is not known whether an immune response might be generated against the  $\beta$ -PDE transgene or  $\beta$ -galactosidase, also possibly resulting in immune-mediated arrest of rescue. We are currently carrying out detailed experiments to answer some of these questions. In addition, the emergence of defective virions allows co-migration of the defective and EAMs in the CsCl preparations. We are presently testing materials such as rubidium chloride and potassium bromide which have higher resolving power than CsCl at  $\rho = 1.3$  (39) in order to obtain purer stocks of EAMs. In summary, we have shown that transgene products can be detected in EAM-injected *rd* retinas for at least 18 weeks post-injection; the  $\alpha$  and  $\beta$  subunits of PDE could be detected up to 90 days postnatal with a maximal PDE activity of 150 nmol/min/mg at 33 days postnatal. The outer nuclear layer of *rd* retinas was rescued for at least 12 weeks post-injection. These results demonstrate an improved level of rescue over the use of first-generation adenoviral vectors and contribute significantly towards the long-term goal of finding a therapy for some retinal and CNS disorders.

## MATERIALS AND METHODS

### Construction of pAd5BPDE, pAd5BM and purification of EAMs

Cloning was carried out using standard techniques and as described previously (7). The murine  $\beta$ -PDE cDNA was obtained as a 2.75 *Eco*RI fragment from pBE1 (40). This cDNA was cloned downstream of a 350 bp human  $\beta$ -PDE promoter (41). The inverted Ad origin of replication and five encapsidation signals were derived from pFG140 (42). The resulting plasmid (Fig. 1) is hereafter referred to as pAd5BPDE. A plasmid (pAd5BN) that was

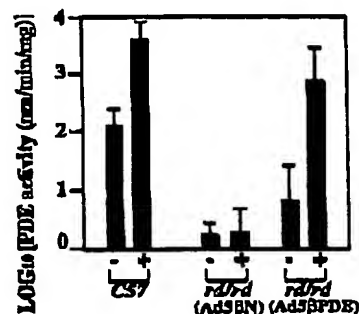


Figure 8. Cyclic GMP-PDE activity following treatment with (+) or without (-) trypsin in Ad5BN or Ad5BPDE injected *rd/rd* retinas at 12 days postnatal. Age-matched CS7BL mice were used as positive controls.

similar in all respects to pAd5BPDE except that it did not contain a  $\beta$ -PDE cDNA was used as a negative control in all experiments. A total of 5  $\mu$ g of pAd5BPDE (or pAd5BN) and 0.5  $\mu$ g linear viral DNA from an E1/E3-deleted virus expressing hpAP were cotransfected into human embryonic kidney 293 cells as described previously (7). Upon cotransfection with helper viral DNA, the circular plasmid pAd5BPDE (or pAd5BN) linearizes, replicates and is packaged along with hpAP into preformed capsids (Ad5BPDE and Ad5BN, respectively). Lysate prepared from cells showing a cytopathic effect at 6–12 days post-transfection was used for serial propagation of EAMs. Purification of virus was achieved by extraction with 1,1,2-trichlorotrifluoroethane and application to CsCl step and self-forming gradients as described previously (7). The EAM-containing band was removed in fractions from the top of the centrifugation tube and CsCl was removed by chromatography on Sephadex G-50. Electron microscopy was performed on uranyl acetate stained EAMs to confirm an intact capsid structure.

### EAM and helper virus titers

Fractions of lysate prepared during various serial passages were used to determine the total titer of the EAM and helper virus prior to subretinal injection. As the EAM and helper virus encode an *E. coli* Lac Z gene and human placental alkaline phosphatase gene, respectively, we performed  $\beta$  galactosidase and alkaline phosphatase assays on infected 293 cells to determine the total titer of the two types of virions. Infected 293 cells were assayed as described previously (7).

### Genomic structure of Ad5BPDE

Approximately  $2 \times 10^9$  Ad5BPDE EAMs were incubated in 0.1 M Tris, pH 8, 2 mM EDTA, 1% SDS, 1 mg/ml Pronase, at 37°C overnight and extracted with phenol-chloroform. A total of 3  $\mu$ g of viral DNA were digested with *Nco*I, fractionated on a 1% agarose gel, electrophoretically transferred onto a nylon membrane (Hybond-N) and hybridized either with a  $^{32}$ P-labeled restriction fragment from the C-terminus of the  $\beta$ -PDE (*Nco*I-Bst1107I fragment from pAd5BPDE) cDNA or the N-terminus of the *E. coli* Lac Z gene (*Not*I-Bst1107I fragment from pAd5BPDE).

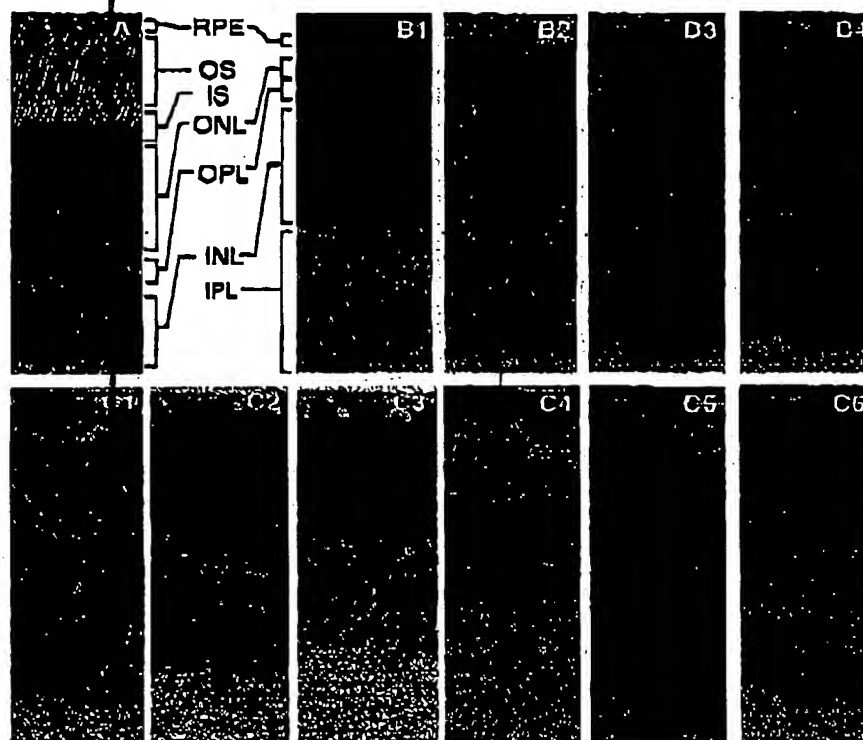


Figure 9. Light micrograph (630 $\times$ ) of Ad5 $\beta$ PDE-injected (C1–C6; 2–12 weeks post-injection in 2 week intervals) or Ad5 $\beta$ N-injected (B1–B4, at 2, 4, 6 and 12 weeks post-injection, respectively) retinas. The outer layers of the adult retina from a C57BL mouse (A) are presented as a control. OS, outer segment; IS, inner segment; OPL, outer plexiform layer; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer. Note that there appears to be no cellular infiltration in the injected retinas.

#### Administration of EAMs

All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology 'Statement for use of animals in ophthalmic and vision research' and in compliance with federal, state and local regulations. C57BL (control) and C57BL *rdle/rdle* mice were bred in our vivarium and reared under 12 h daily cycles of light and darkness. Mice were anesthetized with i.p. administration of 2,2,2-tribromoethanol (0.5 g/kg) between 5 and 6 days postnatal. Injections were performed with a *trans*-scleral, *trans*-choroidal approach (31) using a 33 gauge needle (15° bevel) with a 5  $\mu$ l Hamilton syringe mounted on a micromanipulator. A total of  $3.4 \times 10^7$  purified Ad5 $\beta$ PDE EAMs (or Ad5 $\beta$ N EAMs in the control lateral eye) in 1.0–1.5  $\mu$ l PBS was delivered at a rate of 0.5  $\mu$ l/min.

#### Analyses of $\beta$ -PDE expression

**RT-PCR.** Mice were sacrificed by cervical dislocation and the retinas were dissected rapidly after enucleation. RNA was extracted from Ad5 $\beta$ PDE- or Ad5 $\beta$ N-injected *rd* retinas at 1, 2, 4, 6, 8, 12 and 18 weeks post-injection using TRIzol (Gibco BRL) according to the manufacturer's instructions, and PCR-amplified

with or without prior RT. The RT reaction was carried out in PCR buffer (1 mM each dNTP, 500 mM KCl, 200 mM Tris-HCl, pH 8.4, 25 mM MgCl<sub>2</sub>) with 1 U/ $\mu$ l RNase inhibitor (Perkin Elmer), 100 pmol random hexamers (Amersham), 1  $\mu$ g total RNA, 1 mg/ml nuclease free BSA and 100 U MuLV reverse transcriptase in a final volume of 20  $\mu$ l. The reaction was incubated at 23°C for 10 min and 42°C for 30 min. The RNA-cDNA hybrid was denatured, and reverse transcriptase was inactivated at 95°C for 10 min prior to quick-chill on ice. For cDNA amplification, the RT reaction was brought up to 100  $\mu$ l in 1 $\times$  PCR buffer with 100 ng each oligonucleotide primer (5'-CCACAACCTGTGAGACACG-CAG-3' and 5'-TACGGTTCCTCCAGCTTGTTTCATC-3') and 3 U of Taq polymerase. PCR cycles were performed in a Stratagene robocycler with an initial denaturation at 94°C for 4 min followed by 40 cycles at 94, 55 and 72°C for 1 min each. PCR products were extracted with phenol-chloroform (1:1) and digested with *Dde*I prior to fractionation on agarose gels.

**Immunoblots.** Retinas dissected at the appropriate time from Ad5 $\beta$ PDE or Ad5 $\beta$ N-injected mice were stored at -80°C. Retinas were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 100  $\mu$ M phenylmethylsulfonyl fluoride), centrifuged at 14 000 g for 10 min and the supernatants

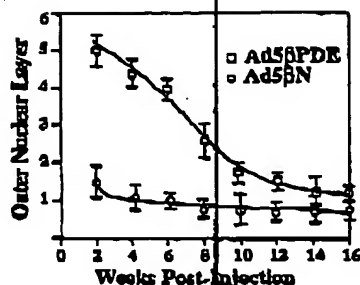


Figure 10. Comparison of outer nuclear layer column height in terms of nuclei in Ad5BPDE- or Ad5BN-injected *rd/rd* retinas in 2 week intervals up to 16 weeks post-injection (117 days postnatal).

were removed and used for protein determination (43) and measurement of cGMP-PDE activity. Proteins (20 µg) were separated by SDS-PAGE utilizing a Tris-glycine buffer system. Additional resolution in the 80–100 kDa range was obtained by modification of a double-inverted gradient acrylamide gel (44). We used three layers: an inverted gradient (6.5–9.5% acrylamide:0.8% bisacrylamide), a separating gel (10.5% acrylamide:0.8% bisacrylamide) and a stacking gel (4% acrylamide:0.8% bisacrylamide). Proteins were transferred to nitrocellulose and probed with a polyclonal antibody (45) to PDE (1:2000 dilution) and visualized using the amplified (secondary biotinylated goat anti-rabbit and tertiary biotin-conjugated alkaline phosphatase) AP kit (Bio-Rad).

**PDE activity analyses.** PDE activity was measured essentially as described previously (46) using a two-step procedure. Briefly, samples (2–5 µg protein) were incubated at 37°C for 15 min with 250 µM [<sup>3</sup>H]cGMP, followed by boiling. After cooling, samples were incubated with 0.4 U alkaline phosphatase at 37°C for 10 min prior to the addition of 1 ml of 1:3 slurry of AG1-X2 resin (Bio-Rad). Samples were left standing at 4°C for 30 min, centrifuged at 3500 g for 20 min, and supernatants were counted. In some experiments PDE was trypsin activated.

#### Histology and β-galactosidase expression

Eyes were enucleated and retinas fixed overnight in 1% formaldehyde and 2% glutaraldehyde. Retinas were immersed in 1% osmium tetroxide for 1 h and dehydrated through a graded series of alcohol. Tissue (2 mm<sup>2</sup> sections) was embedded in araldite and propylene oxide and sectioned at 0.5 µm. Single sections of the retina (extending from the superior ora serrata to the inferior ora serrata) traversing the injection site (and some randomly selected regions of the retina) were studied by light microscopy. Assessment of photoreceptor nuclei was performed in a scheme described previously (38). For analyzing β-galactosidase expression, whole eyes were punctured, washed in PBS and fixed overnight in 0.5% glutaraldehyde. Eyes were washed in PBS and reacted with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) at 1.2 mg/ml in PBS containing 10 mM each of K<sub>3</sub>Fe(CN)<sub>6</sub> and K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, and 2 mM MgCl<sub>2</sub>.



Figure 11. β-Galactosidase staining in Ad5BPDE-injected (A) or PBS-injected (B) eyes from a BALB/c mouse at 3 weeks post-injection. Note the intense staining covering an area of ~1 mm<sup>2</sup> indicated by the arrow.

#### Detection of wild-type virus

As only one recombination event between the hpAP virus and E1 sequences in the 293 cells is required to obtain a replication competent adenovirus (RCA), we evaluated all stocks of EAMs for contamination with RCA by infectivity assay on HeLa cells at a multiplicity of infection of 10 (infection for 12 days). No RCAs were detected throughout our studies.

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